



Technical report

Expressed gene sequence and bioactivity of the IFN γ -response chemokine CXCL11 of swine and cattle

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ABSTRACT

This report describes the cloning and characterization of expressed gene sequences of the swine and bovine interferon-gamma inducible chemokine CXCL11, or I-TAC, associated with type 1 T-helper immune responses, and affirmation of bioactivity of their yeast-expressed protein products. The coding regions of both cDNA sequences were 303 nucleotides in length; each is coded for four exons in the genome. The bovine coding region shared 82% and 70% homology with human and mouse CXCL11, respectively, and the swine coding region 84% and 72% homology, respectively. As expected the swine and bovine CXCL11 sequences showed less homology with other human and mouse C-X-C motif chemokine sequences. Each cDNA was cloned into plasmids and transfected into *Pichia pastoris* (yeast) and the resultant expressed protein purified. Biological activity of each purified chemokine was affirmed by chemotaxis assays. Both swine and bovine CXCL11 were chemotactic for mitogen and IL-2 stimulated peripheral blood mononuclear cells. This is the first report for bioactivity of this chemokine in livestock species. This work provides valuable new reagents for investigating basic immunity as well as vaccine and disease responses in swine and cattle, goals of the U.S. Veterinary Immune Reagent Network which supported this effort.

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The interferon-gamma (IFN γ) inducible chemokines include CXCL9 (MiG), CXCL10 (IP-10), and CXCL11 (I-TAC) and are associated with T-helper 1 (Th1)-type immune responses. All three chemokines mediate responses through a single receptor, CXCR3, the effect of which is to attract type 1 T-helper cells (Loetscher et al., 1996; Cole

et al., 1998). The subject of this report is CXCL11, possibly the most potent of the three CXCR3 ligands (Cole et al., 1998). This chemokine has been implicated in murine experimental autoimmune encephalomyelitis (Klein et al., 2004), pulmonary fibrosis (Burdick et al., 2005), nephrotoxic nephritis (Panzer et al., 2007), and has been shown to play a role in bronchial inflammation in humans (Porter et al., 2008; Costa et al., 2008). CXCL11 has been implicated in protective immunity by recruiting innate NK cell responses (Robertson, 2002) as well as influencing adaptive immune system responses against tumors (Hensbergen et al., 2005; Yang et al., 2006) and viral infections through its ability to attract T effector cells (Piqueras et al., 2006). In swine there is a subset of activated CD4⁺ T cells that express

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CXCR3 (Revilla et al., 2005), the receptor for CXCL9, 10 and 11. CXCR3 is found primarily, but not exclusively, on such activated CD4⁺ T cells (Loetscher et al., 1996).

CXCL11 and its receptor, CXCR3, are likely to be associated with inflammatory diseases of importance to livestock as well as with protective immunity to infectious diseases and tumors. As a tool to begin to investigate their roles in pigs and cattle, we have cloned, sequenced and characterized swine and bovine CXCL11 expressed gene sequences and their yeast-expressed proteins. This work is part of the U.S. Veterinary Immune Reagent Network efforts (US VIRN www.vetimm.org). This is the first report for this chemokine in livestock species.

To generate the clones, the published human CXCL11 messenger RNA sequence (GenBank accession number NM_005409; Cole et al., 1998) was aligned with the *Bos taurus* sequence assembly Btau.3.1 and the NCBI expressed sequence tag (EST) database using the Nucleotide Basic Local Alignment Search Tool (BLASTN; Altschul et al., 1990). The sequence aligned with a predicted bovine mRNA sequence derived from EST data and was identified as “similar to putative alpha chemokine” (GenBank XM_594243) and with a swine EST (GenBank BX914688). The BLASTN program revealed that both these sequences exhibited high homology to known human and mouse CXCL11 sequences. Primers were designed based on the putative full length swine and bovine CXCL11 cDNA sequences as follows:

5'-CAGCAGCAACAAGCATGAGT-3'	(bovine forward primer);
5'-GGTTTCAGATCCTCTTTTCCA-3'	(bovine reverse primer);
5'-ATGGGTGTGAAGGGCATGGGC-3'	(swine forward primer); and
5'-TCATACATTTTGATATCTTAGG-3'	(swine reverse primer).

Total RNA was isolated from PBMC of normal cattle (Blumerman et al., 2007), and from mesenteric lymph nodes, lung, and liver of *Toxoplasma gondii* infected pigs (materials available from an unrelated experiment), using either TRIzol Reagent (Invitrogen, Carlsbad, CA) or RNeasy (QIAGEN, Valencia, CA) according to the manufacturers' protocols. As previously reported (Tompkins et al., 2010) the PCR product was subjected to agarose gel electrophoresis; DNA bands of the predicted size were excised and purified using QIAprep Spin (Invitrogen, Carlsbad, CA). The product was then ligated into pCR2.1-TOPO vector using the TOPO-TA Cloning Kit (Invitrogen) and sequence verified through Genewiz (South Plainfield, NJ). The cDNA sequences for bovine and swine CXCL11 (Fig. 1A) were deposited in GenBank (accession numbers EU276063 and EU682377, respectively). Following optimal alignment of the sequences, the coding regions of the bovine and swine sequences were found to share 82% and 84% identity with that of human CXCL11 (GenBank NM_005409, bases 94–378; Cole et al., 1998), respectively, and 70% and 72% identity with the mouse CXCL11 coding region (GenBank NM_019494, bases 64–366; Widney et al., 2000) (Table 1).

The intron/exon organization of the bovine and swine genes was derived and compared to human, mouse and

swine (Fig. 1B). Alignment of the bovine and swine CXCL11 mRNA sequence with the genomic contigs for each species (GenBank NW_001495216 and CU862040, respectively) using the publicly available program Spidey (Wheelan et al., 2001) revealed that both sequences consist of four exons spanning three introns. Results from Entrez Gene (<http://www.ncbi.nlm.nih.gov/sites/entrez>) map BoCXCL11 to bovine chromosome 6, NC_007304.2 (82672082.82674209). The PoCXCL11 map position to swine chromosome 8 is our prediction; it is not currently annotated as such. The cDNA sequence for both genes was 100% homologous with the putative exon sequences in the genomes.

Translation of the putative coding regions for the bovine and swine cDNA sequences yielded proteins of 100 amino acids in length, with predicted weights of the protein backbones at 10.9 kDa and 11.0 kDa respectively (Fig. 1C). Amino acid identity was determined following optimal alignment of the sequences and subsequently similarity was determined according to the BLOSUM62 substitution matrix for amino acids (Henikoff and Henikoff, 1992). The ClustalW (Felsenstein, 1989; Hall, 1999) alignment of the deduced amino acid sequences for bovine, swine, human, and murine CXCL11 is shown in Fig. 1C. The deduced amino acid sequence of the bovine CXCL11 had 83% identity and 91% similarity with the human protein (GenBank NP_005400) and 57% identity and 78% similarity with the mouse protein (GenBank NP_062367). The predicted swine CXCL11 amino acid sequence displayed 80% identity and 89% similarity with human CXCL11 and 66% identity and 80% similarity to the mouse protein (Table 1). Of particular interest is the fact that the two predicted proteins had conserved cysteine residues found in both the human and mouse proteins. Analysis of the bovine and swine proteins with Signal-P (Emanuelsson et al., 2007) predicted cleavage between amino acids 21 and 22 with over 97% probability; this cleavage site is in agreement with the predictions for the human (Cole et al., 1998) and mouse (Widney et al., 2000) proteins. The coding regions for the putative signal peptides correspond to positions 15 through 77 of the bovine cDNA sequence, and positions 1 through 63 of the swine cDNA sequence.

The nucleotide sequences of the bovine and swine CXCL11 coding regions were compared with sequences encoding other C-X-C motif proteins in mice and humans via ClustalW alignment, and a neighbor phylogenetic tree was constructed using BioEdit software (Felsenstein, 1989; Hall, 1999). The coding sequence for *Bos indicus* CXCL8 (GenBank EU490318) was used as an outlier for the generation of the tree. The resulting tree (Fig. 1D) demonstrated that bovine and swine CXCL11 coding sequences had greater homology with the human CXCL11 coding sequence than does the mouse with human. However, all were more similar to one another than to coding sequences of other members of the C-X-C motif family (image produced through Phylodendron, part of the IUBio-Archive; Gilbert, 1989).

Recombinant expression and purification of bovine and swine CXCL11 proteins was carried out in *Pichia pastoris* at Kingfisher Biotech, Inc. (www.Kingfisherbiotech.com) (Murphy et al., 2001). The CXCL11 DNA encoding the

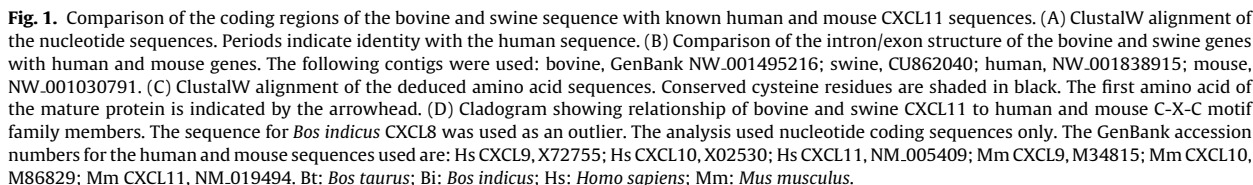


Table 1Comparison of sequences among IFN γ -response chemokines.

Sequence compared to	Nucleotide sequences ^a		Deduced amino acid sequences ^b	
	Bovine CXCL11	Swine CXCL11	Bovine CXCL11	Swine CXCL11
Hs CXCL11	82	84	83/91	80/89
Mm CXCL11	70	72	57/78	66/80
Bt CXCL11	–	87	–	77/88
Ss CXCL11	87	–	77/88	–
Hs CXCL10	54	55	31/55	32/50
Mm CXCL10	50	51	32/55	39/56
Hs CXCL9	44	44	35/56	31/52
Mm CXCL9	43	41	31/56	30/52

^a Values are the percent identity among sequences.^b Values before the slash are percent identity. Values after the slash are percent similarity as determined by the BLOSUM62 amino acid substitution matrix (Henikoff and Henikoff, 1992).

mature protein was subcloned from the pCR2.1-TOPO plasmid into a yeast expression vector under the control of the yeast AOX1 promoter (*Pichia pastoris* Recombinant Protein Expression Kit, Invitrogen Corp., Carlsbad, CA). The expression vector contains the alpha-factor secretion signal from *Saccharomyces cerevisiae* which allows for the secretion of the recombinant protein following electroporation and induction. The recombinant bovine and porcine CXCL11 was then purified by chromatography using Pall AcroSep Strong Cation Exchange columns followed by size exclusion and buffer exchange using Pierce ultrafiltration centrifugal devices (9K). Results have affirmed yields of 1–20 mg purified protein/l of yeast culture. Protein quality and purity were assessed

first by conventional SDS-PAGE gel electrophoresis and Coomassie Blue staining of gels. Recombinant bovine and porcine CXCL11 exhibited two major protein bands, one at approximately 9 kDa (predicted MW) and one larger protein band at approximately 13 kDa. The larger MW protein most likely represents post-translationally modified CXCL11. *P. pastoris* is known to provide post-translational modifications to secreted proteins (gel data available at <http://kingfisherbiotech.blogspot.com>). Protein concentration was determined by absorbance at OD 280. Preparations of rCXCL11 were used for bioassays.

For bioassay of this chemokine a classic Boyden chamber was used for chemotaxis assays (see the US VIRM

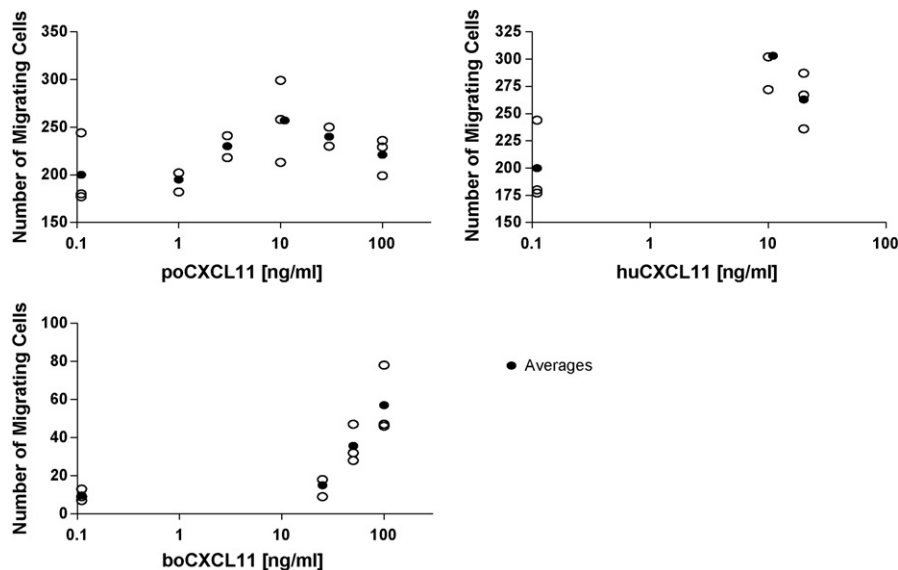


Fig. 2. Activated lymphocyte chemotaxis with yeast-derived rPoCXCL11 and rBoCXCL11 (I-TAC). Pig PBMC were cultured in suboptimal conditions for T cell blast stimulation, i.e., PHA [0.5 μ g/ml] for 3 days, washed and cultured with rHuIL-2 [ProSpec, Israel, 50 unit/ml] for an additional 2 days. Cattle PBMC were stimulated in culture for 3 days with 125 ng/ml rHuIL-2. Once stimulated T cell blasts were harvested and tested for response to yeast-expressed recombinant CXCL11 (Kingfisher rPoCXCL11; Kingfisher rBoCXCL11) or *E. coli*-expressed rHuCXCL11 (ProSpec) in a Boyden Chamber (48 well) using 50 μ l of cell suspension [4.0×10^6 cells/ml] and 25 μ l of medium (control values shown on the abscissa at "0.11" ng/ml = 0) or with various concentrations of rCXCL11. The number of cells that migrated through the membrane after 2.5 h at 37 °C was determined by counting using microscopy. The open circles represent the data from individual wells; the closed circles the average at each concentration. (A) Dose dependent response of activated swine PBMC blasts to various doses of rPoCXCL11. (B) Dose dependent response of activated bovine PBMC blasts to various doses of rHuCXCL11. (C) Dose dependent response of activated bovine PBMC blasts to various doses of rBoCXCL11.

website http://www.umass.edu/vetimm/docs/Bioassay_Protocols.07-30-09.pdf for the detailed chemotaxis protocol). For these assays PBMC were first stimulated with mitogen and interleukin-2 (IL-2). The timing and amount of stimulation was critical to success of chemotaxis. For swine cells, IL-2 by itself was not sufficient to activate the T cells; they required suboptimal PHA prestimulation (Sigma PHA-M 0.5 µg/ml) for 3 days followed by washing, and incubation with rHuIL-2 (ProSpec, Israel, 50 unit/ml) for an additional 2 days to obtain the optimally responsive cells. For bovine cells, IL-2 (R&D Systems, Minn, MN) stimulation at 125 and 250 ng/ml for 3 days resulted in T cells which displayed significant chemotactic responses in several replicate experiments. Fig. 2 shows the chemotaxis results for CXCL11 from three different species assessed in two different labs. For results with pig cells (Fig. 2A) the closed circles represent the average of 2–3 separate wells at each concentration with the medium control reported at 0.11 ng/ml. Swine CXCL11 (Kingfisher yeast rPoCXCL11) gives a typical bell shaped curve; migration of T cell blasts was evident with 3–100 ng/ml of rPoCXCL11. Statistical analyses based on pair wise *t*-test affirmed that migration at 3 and 30 ng/ml was statistically different ($P < 0.10$) from 1 ng/ml, but not medium due to assay variability. Maximal migration was induced at 3–30 ng/ml rPoCXCL11, a dose similar to the response of pig cells to rHuCXCL11 (*Escherichia coli*-expressed by Prospec). For cattle cells statistically ($P < 0.10$) higher migration was found at 50–100 ng/ml with Kingfisher yeast-expressed rBoCXCL11 than with the lower concentrations assessed or with Medium. With human and mouse cells CXCL11 is usually optimal at 10 ng/ml; once CXCL11 starts to saturate the receptor the response is dampened resulting in a bell-shaped curve (Cole et al., 1998). The seemingly greater requirement for migration of bovine cells may reflect a difference in receptor affinity in that species. Nevertheless, these data demonstrate the bioactivity of the US-VIRN recombinant bovine and swine CXCL11.

This work on swine and cattle CXCL11 is evidence of the productivity network developed for the US-VIRN project (www.vetimm.org). These *Pichia* expressed proteins are bioactive and available to the veterinary community through Kingfisher Biotech (website: <http://kingfisherbiotech.com>) for further study of these valuable chemokines. Because of the high level of similarity to the human CXCL11 peptide it is predicted that they will likely function similarly in inflammatory disease processes in ruminants and swine as has been shown for other species. The availability of the reagents will allow this to be evaluated experimentally and monoclonal antibodies for these molecules are in progress which will further enhance experimental evaluation.

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